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NOTE

Requirement of Protein Synthesis for Bacteriophage φX174 Superinfection Exclusion

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Within 5 to 10 min at 37 C, bacteria infected by bacteriophage φX174 acquire the ability to exclude superinfecting φX particles from reproducing themselves. The superinfecting phage are blocked at a stage prior to synthesis of the parental replicative form molecule; the superinfecting deoxyribonucleic acid remains as intact (infective) single strands. Establishment of superinfection exclusion and its maintenance require protein synthesis.

Bacteriophage φX-infected cells acquire the ability, as the infection proceeds, to exclude superinfecting φX particles from reproducing themselves. The physiological basis of this exclusion has been partially described (1, 2). We have used the nonlysing mutant φX*am*3 (cistron E) as the primary infecting phage and then superinfected with $^{32}P^{15}ND$ φX*wt* (wild type). A centrifugal analysis of the intracellular deoxyribonucleic acid (DNA) showed that the superinfecting phage was unable to form parental replicative form (RF) molecules under conditions of exclusion. The superinfecting DNA remains as an infective, and therefore presumably intact, single strand. Superinfecting phage do attach and go into eclipse as measured by plating chloroform-treated infective centers. Cistron E function cannot be performed by excluded phage (as would be expected since no complementary strand is formed to serve as a template for messenger ribonucleic acid synthesis). Exclusion normally takes 5 to 10 min at 37 C to be fully established but is not established or is much delayed in the presence of KCN.

The superinfecting genome is not recovered as free single-stranded DNA unless the cell lysate is phenol-extracted (2). Newbold (Ph.D. Thesis, California Institute of Technology, 1970) has eluted superinfecting viral material from cells. Sedimentation analysis of this material showed that superinfecting phage had been converted to 84*S*-eclipsed phage particles (4). These results suggest that superinfection may be blocked by an alteration of the cell surface which prevents penetration of φX DNA into the cell.

Additional experiments have been performed to determine whether protein synthesis is necessary for the establishment of superinfection exclusion. Strain C was pretreated with chloramphenicol (CAM) at 30 C for 10 min in KC broth. Six cultures were treated, two with 100 μg/ml, two with 30 μg/ml, and two controls with no CAM. One culture at each concentration of CAM was then infected with a multiplicity of of 15 plaque formers of unlabeled φX*am*3*ts*γ*h* (a triple mutant containing an *am* mutation in cistron E, a *ts* mutation in cistron G, and an extended host range mutation). After a 30-min incubation at 30 C, all six cultures were infected with a multiplicity of 0.5 plaque-forming units of $^{32}P^{15}ND$ φX*am*3 (5). The cultures were incubated for an additional 40 min and chilled, the cells were collected by centrifugation and lysed by treatment with lysozyme and versene, and the lysate was phenol-extracted. Each resulting nucleic acid preparation was mixed with a small amount of a viral single-stranded preparation derived from unlabeled φX*ts*γ, brought to a density of 1.72 by the addition of CsCl, and centrifuged to equilibrium at 30,000 rev/min in an SW 39 rotor. The contents of the centrifuge tubes were fractionated by drop collection, and alternate fractions were dried on planchets for the determination of ^{32}P or assayed for infectivity in spheroplasts. Progeny phage from the spheroplast infections were assayed selectively for the various genotypes involved (*am*3*ts*γ*h* was plated on *CR*/φ*X* at 30 C; *am*3 on *HF4714* at 40 C;

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tsγ on *C* at 30 C). The results of these analyses are presented in Fig. 1.

The distribution of parental ^{32}P provides an assay for exclusion. In each gradient, a fraction of the radioactivity bands as free heavy-labeled single strands (peak at fraction 20 to 30). Hybrid density RF has a density nearly the same as light single strands (indicated by the *tsγ* marker at about fraction 50). In the absence of preinfection the majority of parental ^{32}P is found as hybrid RF, either in the absence of CAM or in the presence of 30 or 100 μg of CAM per ml. The preinfected control culture without CAM exhibits exclusion. Less than 10% of the ^{32}P bands at the position of hybrid RF. However, the presence of CAM at either 30 or 100 μg/ml appears to prevent the establishment of exclusion. In the presence of CAM, the majority of parental ^{32}P is converted to hybrid density RF by the preinfected cells.

The infectivity assays give information concerning replication of the viral DNA. In the absence of CAM, progeny single-stranded DNA is formed. Some light *am*3 single strands are apparently formed in the preinfected culture, presumably by the small fraction of parental strands which were not excluded. The amount of *am*3 infectivity found at the light single strand position is reduced by a factor of approximately 10^2 by preinfection. In the presence of 30 μg

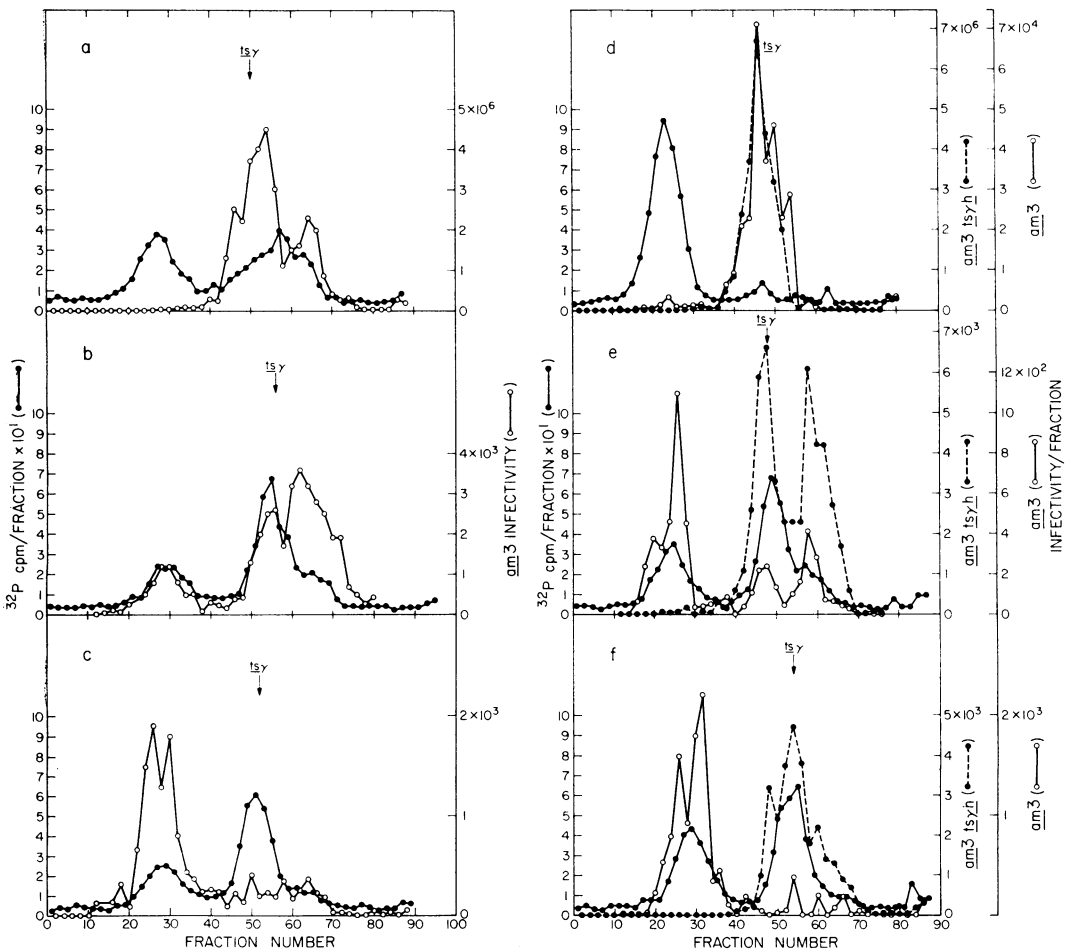

FIG. 1. *Inhibition of superinfection exclusion by chloramphenicol (CAM). CAM treatment, preinfection, and superinfection were performed as described. The distributions of parental ^{32}P (from superinfecting $^{32}P^{15}ND$ φXam3) and the infectivity of am3tsγh (primary infection), am3 (superinfection), and tsγ (light SS marker DNA peak indicated by arrow) are shown after equilibrium CsCl density gradient centrifugation of phenol-extracted DNA. (a) No CAM no preinfection; (b) 30 μg of CAM per ml, no preinfection: (c) 100 μg of CAM per ml, no preinfection; (d) no CAM, preinfection; (e) 30 μg of CAM per ml, preinfection; (f) 100 μg of CAM per ml, preinfection.*

of CAM per ml, the *am*3 RF is able to replicate both in singly infected and in preinfected cells, resulting in a band of fully light RF infectivity. The quantity of replicated RF appears to be smaller in the case of the preinfected culture, presumably because *am*3 RF represents a small fraction of the replicating pool in this case. The quantities of infectivity at the position of the light single-stranded marker suggest that a very small number of single strands may be made in the cultures containing 30 μg of CAM per ml, particularly in the culture without preinfection. [RF is about 20 times less infective per molecule than single-stranded DNA (5).] Detectable replication of RF is not observed in this experiment at 100 μg of CAM per ml.

This experiment demonstrates that CAM treatment can block the establishment of the ability to exclude superinfecting phage from synthesis of parental RF. This is true both at levels of CAM which (almost completely) prevent single-stranded synthesis, but allow RF replication, as well as at CAM concentrations high enough to prevent RF replication. When exclusion is blocked by low levels of CAM, the RF formed by the superinfecting phage is able to replicate. These results suggest that some protein must be formed after infection for exclusion to be established and that the synthesis of this protein is prevented by 30 μg of CAM per ml. This result does not tell us whether the protein is a host or a virus gene product. We can probably eliminate the cistron A product as the causative agent of exclusion, since its synthesis is CAM resistant (3).

A similar type of experiment can be performed to ask whether continued protein synthesis is necessary for the maintenance of superinfection exclusion, once it has been established. C was preinfected with φX*am*3*tsγh* at 30 C. After 30 min of incubation (which is sufficient for exclusion to be established), CAM was added to the culture to give a concentration of 30 μg/ml. After an additional 30 min, the culture was superinfected with $^{32}P^{15}ND$ φX*am*3. The culture was chilled 40 min later, and DNA was prepared and banded as in the previous experiment. Two control experiments were performed at the same time. One sample was simultaneously infected with both phage types at time zero (multiplicities of both phages are the same as in the previous experiment). This sample should not show

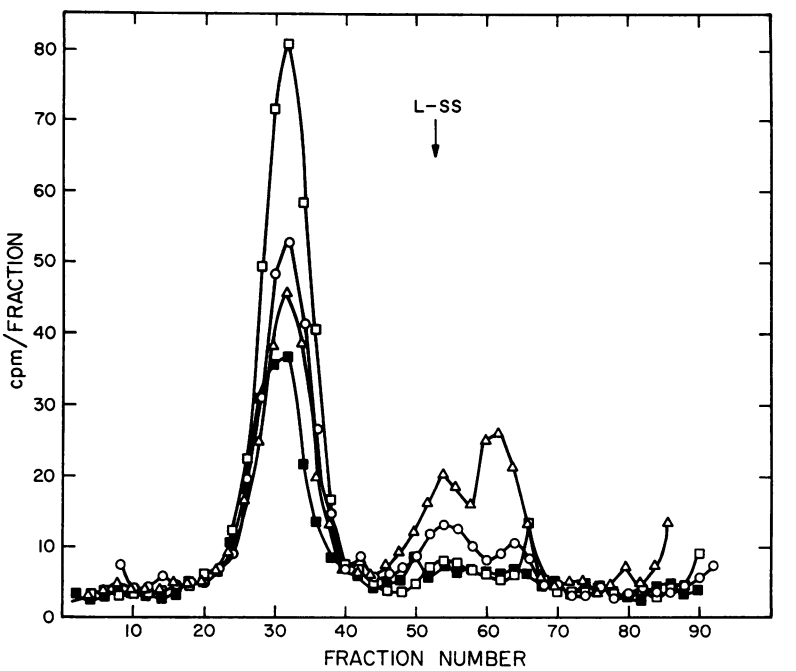

FIG. 2. *Reversal of superinfection exclusion by chloramphenicol (CAM). The infections were performed, and DNA was extracted and banded as described. Distributions of parental ^{32}P after equilibrium CsCl density gradient centrifugation are plotted. Symbols: △, simultaneous infection (no exclusion control); □, superinfection experiment without CAM (exclusion control); ■, superinfection experiment without CAM, eluted with borate-Versene; ○, superinfection after CAM treatment (experimental sample). Patterns have been shifted horizontally so that the tsγ infective single-stranded DNA markers coincide (indicated by arrow L-SS).*

exclusion of the *am*3-labeled phage. Another sample was superinfected 30 min after the primary infection to demonstrate that exclusion actually had been established by the time CAM was added to the experimental culture. Both of these cultures were harvested 40 min after infection with the labeled *am*3. Half of the second control culture was treated with 0.05 M sodium tetraborate and 0.2% Versene to see whether excluded phage DNA remains extracellular and can therefore be eluted by this procedure (2). The cells were collected by centrifugation and resuspended, the supernatant solution and the resuspended cells were counted to determine the distribution of ^{32}P, and DNA was prepared from the cells. The resulting distributions of radioactivity after equilibrium centrifugation in CsCl are shown in Fig. 2. The results suggest that CAM can at least partially reverse superinfection exclusion, since the amount of ^{32}P at the position of hybrid RF in the CAM-treated sample is intermediate between the two types of control samples. The reason for the broadness and splitting of the ^{32}P band at the hybrid RF position is not known. It may be some kind of artifact due to viscosity of cellular DNA which bands near this position; however, the *ts*γ single-stranded marker peaks were symmetrical. A fraction (about 25 to 30%) of the excluded phage DNA was removed by the borate-Versene treatment, indication that at least part of the parental DNA remains extracellular.

Apparently, on the basis of these results, continued protein synthesis is necessary to maintain complete exclusion. The nature of the protein involved is not known. One possibility is a protein which alters the cell surface in such a way that the φX injection process (4) is blocked. One might suppose, alternatively, that the incoming viral single strand is blocked from the formation of RF by forming a complex with free-phage coat protein. Density labeling experiments (2) have shown, however, that superinfecting DNA does not appear in mature phage particles coated with newly synthesized protein. The proposed complex must therefore be an incomplete particle. In CAM the pool of coat protein could be depleted, allowing superinfecting phage now to form RF. To test this idea, and the more general idea that the product of one of the known φX cistrons is necessary for exclusion, mutants in all eight cistrons should be tested to see whether they can establish exclusion.

These results are drawn from a thesis submitted by C. A. H. to the California Institute of Technology in partial fulfillment of the requirements for the Ph.D. degree.

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